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(71) Applicant: CHIRON CORPORATION [US/US]; 4560 Horton Street – R440, Emeryville, CA 94608 (US).

(72) Inventors: HOUGTON, Michael; Chiron Corporation, 4560 Horton Street – R440, Emeryville, CA 94608 (US). CHOO, Qui-Lim; Chiron Corporation, 4560 Horton Street – R440, Emeryville, CA 94608 (US). ABRIGNANI, Sergio; Chiron Corporation, 4560 Horton Street – R440, Emeryville, CA 94608 (US). CHIEN, David; Chiron Corporation, 4560 Horton Street – R440, Emeryville, CA 94608 (US). SELBY, Mark; Chiron Corporation, 4560 Horton Street – R440, Emeryville, CA 94608 (US). GLAZER, Edward; Chiron Corporation, 4560 Horton Street – R440, Emeryville, CA 94608 (US).

(74) Agents: HARBIN, Alisa, A.; Chiron Corporation, Intellectual Property – R440, P.O. Box 8097, Emeryville, CA 49662-8097 (US) et al.

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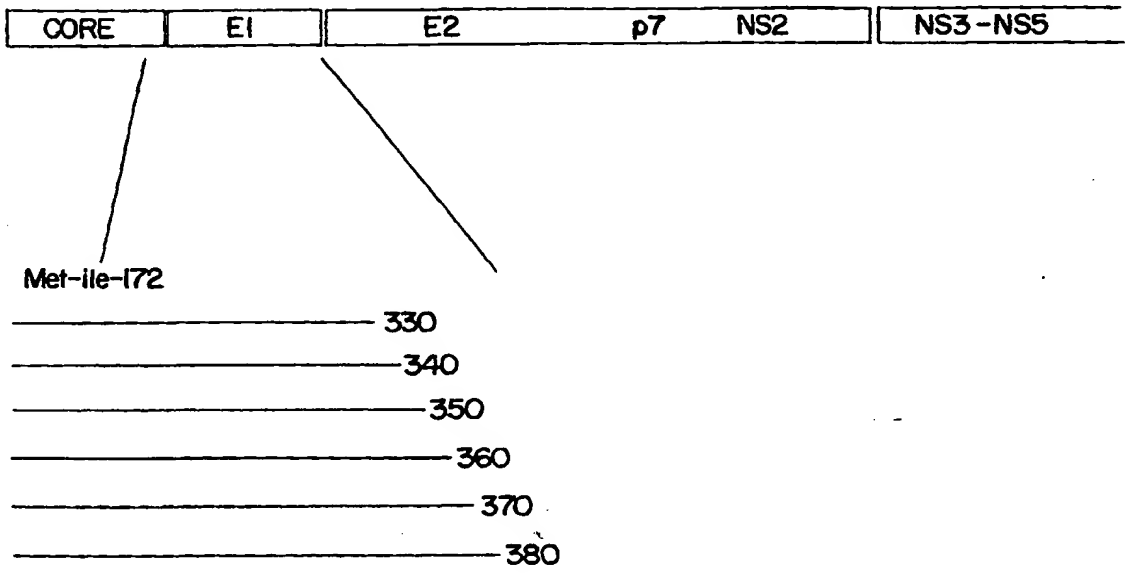

(57) Abstract

Methods for obtaining recombinantly produced, C-terminally truncated, E1 and E2 polypeptides from cell lysates are disclosed. The intracellularly expressed truncated molecules display improved biological properties as compared to their secreted counterparts.

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INTRACELLULAR PRODUCTION OF HEPATITIS C E1 AND E2 TRUNCATED POLYPEPTIDES

Background of the Invention

Technical Field

The present invention pertains generally to viral proteins. In particular, the invention relates to improved methods for isolating truncated forms of hepatitis C virus E1 and E2 proteins having improved biological properties for use in vaccine compositions and as diagnostic reagents.

Background of the Invention

Hepatitis C Virus (HCV) is the principal cause of parenteral non-A, non-B hepatitis which is transmitted largely through blood transfusion and sexual contact. The virus is present in 0.4 to 2.0% of blood donors. Chronic hepatitis develops in about 50% of infections and of these, approximately 20% of infected individuals develop liver cirrhosis which sometimes leads to hepatocellular carcinoma. Accordingly, the study and control of the disease is of medical importance.

The viral genomic sequence of HCV is known, as are methods for obtaining the sequence. See, e.g., International Publication Nos. WO 89/04669; WO 90/11089; and WO 90/14436. HCV has a 9.5 kb positive-sense, single-stranded RNA genome and is a member of the Flaviridae family of viruses. At least six distinct, but related genotypes of HCV, based on phylogenetic analyses, have been identified (Simmonds

et al., *J. Gen. Virol.* (1993) 74:2391-2399). The virus encodes a single polyprotein having more than 3000 amino acid residues (Choo et al., *Science* (1989) 244:359-362; Choo et al., *Proc. Natl. Acad. Sci. USA* (1991) 88:2451-2455; Han et al., *Proc. Natl. Acad. Sci. USA* (1991) 88:1711-1715). The polyprotein is processed co- and post-translationally into both structural and non-structural (NS) proteins.

In particular, there are three putative structural proteins, consisting of the N-terminal nucleocapsid protein (termed "core") and two envelope glycoproteins, "E1" (also known as E) and "E2" (also known as E2/NS1). (See, Houghton et al., *Hepatology* (1991) 14:381-388, for a discussion of HCV proteins, including E1 and E2.) E1 is detected as a 32-35 kDa species and is converted into a single endo H-sensitive band of approximately 18 kDa. By contrast, E2 displays a complex pattern upon immunoprecipitation consistent with the generation of multiple species (Grakoui et al., *J. Virol.* (1993) 67:1385-1395; Tomei et al., *J. Virol.* (1993) 67:4017-4026.). The HCV E1 and E2 glycoproteins are of considerable interest because they have been shown to be protective in primate studies. (Choo et al., *Proc. Natl. Acad. Sci. USA* (1994) 91:1294-1298).

Full-length E1 and E2 are retained within cells and have been shown to lack complex carbohydrate when expressed stably or in a transient Vaccinia virus system (Spaete et al., *Virology* (1992) 188:819-830; Ralston et al., *J. Virol.* (1993) 67:6753-6761). Since the E1 and E2 proteins are normally membrane-bound in these expression systems, experimenters had previously thought it desirable to produce secreted forms to facilitate purification of the proteins for further use.

For example, an HCV E2 molecule, truncated at amino acid 661 and which is secreted from mammalian cells, has been described. Spaete et al., *Virology* (1992) 188:819-830. The production of truncated, secreted HCV E1 and E2 molecules has also been disclosed in International Publication No. WO 96/04301, published February 15, 1996. Inudoh et al., *Vaccine* (1996) 14:1590-1596, describes the production of an HCV E2 molecule lacking the C-terminal hydrophobic domain. This protein was secreted into culture medium and found to be more antigenic than intracellularly produced counterparts.

Depending on the expression system used, such secreted proteins may not retain the native conformation and may include modified glycosylation patterns. Thus, purification of intracellularly produced HCV E1 and E2 proteins has been attempted in order to preserve the native conformation of the proteins. See, e.g., International Publication No. WO 92/08734, published May 29, 1992.

Despite the above attempts at obtaining HCV E1 and E2, a need still exists for alternative methods of efficiently purifying immunogenic HCV E1 and E2 molecules for use in vaccine compositions and as diagnostic reagents.

Summary of the Invention

The present invention is based on the isolation of HCV E1 and E2 proteins which display improved biological properties. The proteins are truncated and can be produced using recombinant techniques. Such truncated proteins are normally secreted into culture medium. However, the proteins for use herein are isolated from the cells rather than from culture medium. The molecules so isolated display enhanced receptor-binding abilities, perform

better in assays designed to measure the ability of proteins to elicit the production of HCV neutralizing antibodies; and are more immunoreactive and therefore provide improved diagnostic reagents, as compared to their secreted counterparts.

Accordingly, in one embodiment, the subject invention is directed to a method for isolating an HCV E1 polypeptide that lacks a portion of its C-terminus beginning at about amino acid 370, numbered with reference to the HCV1 E1 amino acid sequence. The method comprises:

(a) providing a population of host cells transformed with a polynucleotide comprising a coding sequence for the HCV E1 polypeptide, wherein the coding sequence is operably linked to control elements such that the coding sequence can be transcribed and translated in the host cell;

(b) culturing the population of cells under conditions whereby the HCV E1 polypeptide is expressed intracellularly;

(c) disrupting the host cells; and

(d) isolating the HCV E1 polypeptide from the disrupted cells.

In particularly preferred embodiments, the HCV E1 polypeptide produced by the method lacks a portion of its C-terminus beginning at about amino acid 360, numbered with reference to the HCV1 E1 amino acid sequence.

In another embodiment, the invention is directed to a method for isolating an HCV E2 polypeptide that lacks a portion of its C-terminus beginning at about amino acid 730 but not extending beyond about amino acid 625, numbered with reference to the HCV1 E2 amino acid sequence. The method comprises:

(a) providing a population of host cells transformed with a polynucleotide comprising a coding sequence for the HCV E2 polypeptide, wherein the coding sequence is operably linked to control elements such that the coding sequence can be transcribed and translated in the host cell;

(b) culturing the population of cells under conditions whereby the HCV E2 polypeptide is expressed intracellularly;

(c) disrupting the host cells; and

(d) isolating the HCV E2 polypeptide from the disrupted cells.

In particularly preferred embodiments, the HCV E2 polypeptide produced by the method lacks at least a portion of its C-terminus beginning at about amino acid 725, numbered with reference to the HCV1 E2 amino acid sequence, and more particularly, lacks a portion of its C-terminus beginning at about amino acid 715, 661 or 655.

Further embodiments of the subject invention pertain to HCV E1 and HCV E2 polypeptides produced by the above methods, as well as vaccine compositions comprising the HCV polypeptides and methods of preparing the vaccine compositions.

In yet other embodiments, the invention is directed to methods of detecting the presence or absence of HCV infection in a subject suspected of having an HCV infection. The methods comprise:

(a) providing a biological sample from the subject;

(b) providing an HCV E1 polypeptide or an HCV E2 polypeptide, as described above; and

(c) contacting the biological sample with the HCV polypeptide, under conditions which allow HCV antibodies, if present in the biological sample, to bind with the HCV polypeptide,

thereby detecting the presence or absence of HCV infection in the subject.

In other embodiments, the invention is directed to immunodiagnostic test kits for detecting HCV infection. The test kits include an HCV E1 or HCV E2 polypeptide, as described above, and instructions for conducting the immunodiagnostic test.

These and other embodiments of the present invention will readily occur to those of ordinary skill in the art in view of the disclosure herein.

Brief Description of the Figures

Figure 1 shows the full-length nucleotide sequence and corresponding amino acid sequence for HCV1 E1 which includes the N-terminal signal sequence and the C-terminal membrane anchor domain.

Figures 2A-2C show the full-length nucleotide sequence and the corresponding amino acid sequence for the HCV1 E2/NS2 region which includes the N-terminal signal sequence for E2 and the C-terminal membrane anchor domain for E2.

Figure 3 depicts the HCV E1 cDNA templates described in the Examples. The core through NS2 region is shown on the top and is drawn to scale; the distal NS3 through NS5 is not drawn to scale. The E1 region has been expanded to better display the various templates. The numbers to the right refer to the amino acid endpoint used in each template.

Figure 4 depicts some of the HCV E2 cDNA templates described in the Examples. The core through NS2 region is shown on the top and is drawn to scale; the distal NS3 through NS5 is not drawn to scale. The E2/NS2 region has been expanded to better display the various templates. The column to the left refers to the amino acid endpoint used in each template.

Figure 5 depicts the results of a neutralization of binding assay performed with secreted, truncated E2 and intracellularly produced (internal), truncated E2.

Detailed Description of the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional methods of virology, microbiology, molecular biology and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, et al. *Molecular Cloning: A Laboratory Manual* (2nd Edition, 1989); *DNA Cloning: A Practical Approach*, vol. I & II (D. Glover, ed.); *Animal Cell Culture* (R. Freshney, ed., 1986); Perbal, *A Practical Guide to Molecular Cloning* (1984); *Fundamental Virology*, 2nd Edition, vol. I & II (B.N. Fields and D.M. Knipe, eds.); *Protein Purification Applications: A Practical Approach*, (E.L.V. Harris and S. Angal, Eds., 1990); and T.E. Creighton, *Proteins: Structures and Molecular Properties* (W.H. Freeman and Company, 1993).

As used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural references unless the content clearly dictates otherwise.

I. Definitions

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

By an "E1 polypeptide" is meant a molecule derived from an HCV E1 region. The mature E1 region

of HCV1 begins at approximately amino acid 192 of the polyprotein and continues to approximately amino acid 383 (see Figure 1). Amino acids at around 173 through approximately 191 serve as a signal sequence for E1. Thus, by an "E1 polypeptide" is meant either a precursor E1 protein, including the signal sequence, or a mature E1 polypeptide which lacks this sequence, or even an E1 polypeptide with a heterologous signal sequence. The E1 polypeptide includes a C-terminal membrane anchor sequence which occurs at approximately amino acid positions 360-383 (see, International Publication No. WO 96/04301, published February 15, 1996).

By an "E2 polypeptide" is meant a molecule derived from an HCV E2 region. The mature E2 region of HCV1 begins at approximately amino acid 383-385 (see Figure 2). A signal peptide begins at approximately amino acid 364 of the polyprotein. Thus, by an "E2 polypeptide" is meant either a precursor E2 protein, including the signal sequence, or a mature E2 polypeptide which lacks this sequence, or even an E2 polypeptide with a heterologous signal sequence. The E2 polypeptide includes a C-terminal membrane anchor sequence which occurs at approximately amino acid positions 715-730 and may extend as far as approximately amino acid residue 746 (see, Lin et al., *J. Virol.* (1994) 68:5063-5073).

Representative E1 and E2 regions from HCV1 are shown in Figures 1 and 2, respectively. For purposes of the present invention, the E1 and E2 regions are defined with respect to the amino acid number of the polyprotein encoded by the genome of HCV1, with the initiator methionine being designated position 1. However, it should be noted that the term an "E1 polypeptide" or an "E2 polypeptide" as used herein is not limited to the HCV1 sequence. In this

regard, the corresponding E1 or E2 regions in another HCV isolate can be readily determined by aligning sequences from the two isolates in a manner that brings the sequences into maximum alignment. This can be performed with any of a number of computer software packages, such as ALIGN 1.0, available from the University of Virginia, Department of Biochemistry (Attn: Dr. William R. Pearson). See, Pearson et al., *Proc. Natl. Acad. Sci. USA* (1988) 85:2444-2448.

Furthermore, an "E1 polypeptide" or an "E2 polypeptide" as defined herein is not limited to a polypeptide having the exact sequence depicted in the Figures. Indeed, the HCV genome is in a state of constant flux and contains several variable domains which exhibit relatively high degrees of variability between isolates. It is readily apparent that the terms encompass E1 and E2 polypeptides from any of the various HCV isolates including isolates having any of the 6 genotypes of HCV described in Simmonds et al., *J. Gen. Virol.* (1993) 74:2391-2399), as well as newly identified isolates, and subtypes of these isolates, such as HCV1a, HCV1b etc.

Additionally, the terms "E1 polypeptide" and "E2 polypeptide" encompass proteins which include additional modifications to the native sequence, such as additional internal deletions, additions and substitutions (generally conservative in nature). These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through naturally occurring mutational events. All of these modifications are encompassed in the present invention so long as the modified E1 and E2 polypeptides function for their intended purpose. Thus, for example, if the E1 and/or E2 polypeptides are to be used in vaccine compositions, the modifications must be such that immunological activity

(i.e., the ability to elicit an antibody response to the polypeptide) is not lost. Similarly, if the polypeptides are to be used for diagnostic purposes, such capability must be retained.

An E1 or E2 polypeptide "lacking all or a portion of its membrane spanning domain" is an E1 or E2 polypeptide, respectively, as defined above, which has been manipulated to delete all or a part of the membrane anchor sequence which functions to associate the polypeptide to the endoplasmic reticulum. Normally, such a polypeptide is capable of secretion into growth medium in which an organism expressing the protein is cultured. However, for purposes of the present invention, such polypeptides may also be recovered intracellularly. Secretion into growth media is readily determined using a number of detection techniques, including, e.g., polyacrylamide gel electrophoresis and the like, and immunological techniques such as immunoprecipitation assays as described in, e.g., International Publication No. WO 96/04301, published February 15, 1996. With E1, generally polypeptides terminating with about amino acid position 370 and higher (based on the numbering of HCV1 E1) will be retained by the ER and hence not secreted into growth media. With E2, polypeptides terminating with about amino acid position 731 and higher (also based on the numbering of the HCV1 E2 sequence) will be retained by the ER and not secreted. (See, e.g., International Publication No. WO 96/04301, published February 15, 1996). It should be noted that these amino acid positions are not absolute and may vary to some degree.

Although not all possible C-terminal truncations have been exemplified herein, it is to be understood that intervening truncations, such as e.g., E1 polypeptides ending in amino acids 351, 352, 353

and so on, or E2 polypeptides ending in for example amino acids 716, 717, 718 and so on, are also encompassed by the present invention. Hence, all E1 polypeptides, terminating at about amino acids 369 and lower, and all E2 polypeptides, terminating at about amino acids 730 and lower, are intended to be captured by the present invention.

Furthermore, the C-terminal truncation can extend beyond the transmembrane spanning domain towards the N-terminus. Thus, for example, E1 truncations occurring at positions lower than, e.g., 360 and E2 truncations occurring at positions lower than, e.g., 715, are also encompassed by the present invention. All that is necessary is that the truncated E1 and E2 polypeptides remain functional for their intended purpose. However, particularly preferred E1 constructs are those that do not extend beyond about amino acid 300. Preferred E2 constructs are those with C-terminal truncations that do not extend beyond about amino acid position 500. Particularly preferred E2 truncations are those molecules truncated after about amino acid 715, 661 or 655.

An E1 and/or E2 polypeptide is produced "intracellularly" when it is found within the cell, either associated with components of the cell, such as in association with the endoplasmic reticulum (ER) or the Golgi Apparatus, or when it is present in the soluble cellular fraction. The E1 and/or E2 polypeptides of the present invention may also be secreted into growth medium so long as sufficient amounts of the polypeptides remain present within the cell such that they can be purified from cell lysates using techniques described herein.

An "immunogenic" HCV E1 or E2 protein is a molecule that includes at least one epitope such that

the molecule is capable of either eliciting an immunological reaction in an individual to which the protein is administered or, in the diagnostic context, is capable of reacting with antibodies directed against the HCV in question.

By "epitope" is meant a site on an antigen to which specific B cells and/or T cells respond, rendering the molecule including such an epitope capable of eliciting an immunological reaction or capable of reacting with HCV antibodies present in a biological sample. The term is also used interchangeably with "antigenic determinant" or "antigenic determinant site." An epitope can comprise 3 or more amino acids in a spatial conformation unique to the epitope. Generally, an epitope consists of at least 5 such amino acids and, more usually, consists of at least 8-10 such amino acids. Methods of determining spatial conformation of amino acids are known in the art and include, for example, x-ray crystallography and 2-dimensional nuclear magnetic resonance. Furthermore, the identification of epitopes in a given protein is readily accomplished using techniques well known in the art, such as by the use of hydrophobicity studies and by site-directed serology. See, also, Geysen et al., *Proc. Natl. Acad. Sci. USA* (1984) 81:3998-4002 (general method of rapidly synthesizing peptides to determine the location of immunogenic epitopes in a given antigen); U.S. Patent No. 4,708,871 (procedures for identifying and chemically synthesizing epitopes of antigens); and Geysen et al., *Molecular Immunology* (1986) 23:709-715 (technique for identifying peptides with high affinity for a given antibody). Antibodies that recognize the same epitope can be identified in a simple immunoassay showing the ability of one antibody to block the binding of another antibody to a target antigen.

An "immunological response" as used herein is the development in the subject of a humoral and/or a cellular immune response to the E1 and/or E2 polypeptide when the polypeptide is present in a vaccine composition.

These antibodies may also neutralize infectivity, and/or mediate antibody-complement or antibody dependent cell cytotoxicity to provide protection to an immunized host. Immunological reactivity may be determined in standard immunoassays, such as a competition assays, well known in the art.

Two polynucleotides or protein molecules are "substantially homologous" when at least about 40-50%, preferably at least about 70-80%, and most preferably at least about 85-95%, of the nucleotides or amino acids from the molecules match over a defined length of the molecule. As used herein, substantially homologous also refers to molecules having sequences which show identity to the specified nucleic acid or protein molecule. Nucleic acid molecules that are substantially homologous can be identified in a Southern hybridization experiment under, for example, stringent conditions, as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Sambrook et al., *supra*; *DNA Cloning*, vols I & II, *supra*; *Nucleic Acid Hybridization*, *supra*. For example, homology can be determined by hybridization of polynucleotides under conditions which form stable duplexes between homologous regions. Stable duplexes are those, for example, which withstand digestion with a single-stranded specific nuclease(s), such as S1. Such duplexes can be analyzed by various methods, such as size determination of digested fragments.

"Stringency" refers to conditions in a hybridization reaction that favor association of very

similar sequences over sequences that differ. For example, the combination of temperature and salt concentration should be chosen that is approximately 12 to 20 degrees C below the calculated Tm of the hybrid under study.

Other techniques for determining sequence identity are well known in the art and include determining the sequence of the polynucleotide or polypeptide of interest and comparing this to a second sequence. Programs available in the Wisconsin Sequence Analysis Package, Version 8 (available from Genetics Computer Group, Madison, WI) for example, the BESTFIT, FASTA and GAP programs, are capable of calculating identity between two molecules.

An "isolated" or "purified" protein or polypeptide is a protein which is separate and discrete from a whole organism with which the protein is normally associated in nature. It is apparent that the term denotes proteins of various levels of purity. Typically, a composition containing a purified protein will be one in which at least about 35%, preferably at least about 40-50%, more preferably, at least about 75-85%, and most preferably at least about 90% or more, of the total protein in the composition will be the protein in question.

A "coding sequence" or a sequence which "encodes" a selected protein, is a nucleic acid sequence which is transcribed (in the case of DNA) and translated (in the case of mRNA) into a polypeptide *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5′ (amino) terminus and a translation stop codon at the 3′ (carboxy) terminus. A coding sequence can include, but is not limited to cDNA from viral nucleotide sequences as well as synthetic and

semisynthetic DNA sequences and sequences including base analogs. A transcription termination sequence may be located 3' to the coding sequence.

"Control elements" refers collectively to promoter sequences, ribosome binding sites, polyadenylation signals, transcription termination sequences, upstream regulatory domains, enhancers, and the like, which collectively provide for the transcription and translation of a coding sequence in a host cell. Not all of these control elements need always be present so long as the desired gene is capable of being transcribed and translated.

A control element "directs the transcription" of a coding sequence in a cell when RNA polymerase will bind the promoter sequence and transcribe the coding sequence into mRNA, which is then translated into the polypeptide encoded by the coding sequence.

"Operably linked" refers to an arrangement of elements wherein the components so described are configured so as to perform their usual function. Thus, control elements operably linked to a coding sequence are capable of effecting the expression of the coding sequence when RNA polymerase is present. The control elements need not be contiguous with the coding sequence, so long as they function to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between, e.g., a promoter sequence and the coding sequence and the promoter sequence can still be considered "operably linked" to the coding sequence.

"Recombinant" as used herein to describe a nucleic acid molecule means a polynucleotide of genomic, cDNA, semisynthetic, or synthetic origin which, by virtue of its origin or manipulation: (1) is not associated with all or a portion of the

polynucleotide with which it is associated in nature; and/or (2) is linked to a polynucleotide other than that to which it is linked in nature. The term "recombinant" as used with respect to a protein or polypeptide means a polypeptide produced by expression of a recombinant polynucleotide. "Recombinant host cells," "host cells," "cells," "cell lines," "cell cultures," and other such terms denoting procaryotic microorganisms or eucaryotic cell lines cultured as unicellular entities, are used interchangeably, and refer to cells which can be, or have been, used as recipients for recombinant vectors or other transfer DNA, and include the progeny of the original cell which has been transfected. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement to the original parent, due to accidental or deliberate mutation. Progeny of the parental cell which are sufficiently similar to the parent to be characterized by the relevant property, such as the presence of a nucleotide sequence encoding a desired peptide, are included in the progeny intended by this definition, and are covered by the above terms.

By "vertebrate subject" is meant any member of the subphylum chordata, including, without limitation, humans and other primates, including non-human primates such as chimpanzees and other apes and monkey species; farm animals such as cattle, sheep, pigs, goats and horses; domestic mammals such as dogs and cats; laboratory animals including rodents such as mice, rats and guinea pigs; birds, including domestic, wild and game birds such as chickens, turkeys and other gallinaceous birds, ducks, geese, and the like. The term does not denote a particular age. Thus, both

adult and newborn individuals are intended to be covered.

As used herein, a "biological sample" refers to a sample of tissue or fluid isolated from an individual, including but not limited to, for example, blood, plasma, serum, fecal matter, urine, bone marrow, bile, spinal fluid, lymph fluid, samples of the skin, external secretions of the skin, respiratory, intestinal, and genitourinary tracts, samples derived from the gastric epithelium and gastric mucosa, tears, saliva, milk, blood cells, organs, biopsies and also samples of *in vitro* cell culture constituents including but not limited to conditioned media resulting from the growth of cells and tissues in culture medium, e.g., recombinant cells, and cell components.

The terms "label" and "detectable label" refer to a molecule capable of detection, including, but not limited to, radioactive isotopes, fluorescers, chemiluminescers, enzymes, enzyme substrates, enzyme cofactors, enzyme inhibitors, chromophores, dyes, metal ions, metal sols, ligands (e.g., biotin or haptens) and the like. The term "fluorescer" refers to a substance or a portion thereof which is capable of exhibiting fluorescence in the detectable range. Particular examples of labels which may be used with the invention include, but are not limited to fluorescein, rhodamine, dansyl, umbelliferone, Texas red, luminol, acradimum esters, NADPH, α-β-galactosidase, horseradish peroxidase, glucose oxidase, alkaline phosphatase and urease.

II. Modes of Carrying Out the Invention

The present invention is based on the discovery of novel methods for obtaining recombinantly produced, C-terminally truncated, E1 and E2

polypeptides from cell lysates, rather than directly from growth media. As demonstrated herein, the molecules purified in this manner have surprisingly better biological properties than their secreted counterparts. For example, the intracellularly produced molecules display enhanced receptor-binding abilities, exhibit superior performance in assays designed to measure the ability of proteins to elicit the production of HCV neutralizing antibodies, and are more immunoreactive and therefore provide improved diagnostic reagents, as compared to their secreted counterparts.

While not wishing to be bound by any particular theory, the intracellularly expressed forms of HCV E1 and E2 may more closely resemble the native viral proteins due to the carbohydrate motifs present on the molecules, while the secreted glycoproteins may contain modified carbohydrate moieties or glycosylation patterns. Furthermore, the intracellularly produced forms of E1 and E2 may be conformationally different than the secreted forms.

In particular, a number of C-terminally truncated E1 and E2 polypeptides, lacking portions of the membrane spanning domain, have been constructed. See Figures 3 and 4, respectively, and the examples. As shown in the examples, a truncated construct terminating at amino acid 715 and termed "$E2_{715}$" herein, is surprisingly more immunoreactive when purified from recombinant cells, than $E2_{715}$ secreted into, and purified from, media. Furthermore, molecules terminating at amino acids 661 and 655 also show enhanced immunoreactivity. Thus, the intracellularly produced truncated HCV polypeptides are excellent candidates for vaccines and diagnostics.

The intracellularly produced, truncated E1 and E2 polypeptides can be obtained using a variety of

techniques. For example, the polypeptides can be generated using recombinant techniques, well known in the art. In this regard, oligonucleotide probes can be devised based on the known sequences of the HCV genome and used to probe genomic or cDNA libraries for E1 and E2 genes. The genes can then be further isolated using standard techniques and, e.g., restriction enzymes employed to truncate the gene at desired portions of the full-length sequence. Similarly, the E1 and E2 genes can be isolated directly from cells and tissues containing the same, using known techniques, such as phenol extraction and the sequence further manipulated to produce the desired truncations. *See, e.g.*, Sambrook et al., *supra*, for a description of techniques used to obtain and isolate DNA. Finally, the genes encoding the truncated E1 and E2 polypeptides can be produced synthetically, based on the known sequences. The nucleotide sequence can be designed with the appropriate codons for the particular amino acid sequence desired. In general, one will select preferred codons for the intended host in which the sequence will be expressed. The complete sequence is generally assembled from overlapping oligonucleotides prepared by standard methods and assembled into a complete coding sequence. *See, e.g.*, Edge (1981) *Nature* 292:756; Nambair *et al.* (1984) *Science* 223:1299; Jay *et al.* (1984) *J. Biol. Chem.* 259:6311.

Once coding sequences for the desired proteins have been isolated or synthesized, they can be cloned into any suitable vector or replicon for expression. Numerous cloning vectors are known to those of skill in the art, and the selection of an appropriate cloning vector is a matter of choice. Examples of recombinant DNA vectors for cloning and host cells which they can transform include the

bacteriophage λ (*E. coli*), pBR322 (*E. coli*), pACYC177 (*E. coli*), pKT230 (gram-negative bacteria), pGV1106 (gram-negative bacteria), pLAFR1 (gram-negative bacteria), pME290 (non-*E. coli* gram-negative bacteria), pHV14 (*E. coli* and *Bacillus subtilis*), pBD9 (*Bacillus*), pIJ61 (*Streptomyces*), pUC6 (*Streptomyces*), YIp5 (*Saccharomyces*), YCp19 (*Saccharomyces*) and bovine papilloma virus (mammalian cells). *See, generally, DNA Cloning*: Vols. I & II, *supra*; Sambrook *et al.*, *supra*; B. Perbal, *supra*.

Insect cell expression systems, such as baculovirus systems, can also be used and are known to those of skill in the art and described in, *e.g.*, Summers and Smith, *Texas Agricultural Experiment Station Bulletin No. 1555* (1987). Materials and methods for baculovirus/insect cell expression systems are commercially available in kit form from, *inter alia*, Invitrogen, San Diego CA ("MaxBac" kit).

Plant expression systems can also be used to produce the truncated E1 and E2 proteins. Generally, such systems use virus-based vectors to transfect plant cells with heterologous genes. For a description of such systems see, e.g., Porta et al., *Mol. Biotech.* (1996) 5:209-221; and Hackland et al., *Arch. Virol.* (1994) 139:1-22.

Viral systems, such as a vaccinia based infection/transfection system, as described in Tomei et al., *J. Virol.* (1993) 67:4017-4026 and Selby et al., *J. Gen. Virol.* (1993) 74:1103-1113, will also find use with the present invention. In this system, cells are first transfected *in vitro* with a vaccinia virus recombinant that encodes the bacteriophage T7 RNA polymerase. This polymerase displays exquisite specificity in that it only transcribes templates bearing T7 promoters. Following infection, cells are transfected with the DNA of interest, driven by a T7

promoter. The polymerase expressed in the cytoplasm from the vaccinia virus recombinant transcribes the transfected DNA into RNA which is then translated into protein by the host translational machinery. The method provides for high level, transient, cytoplasmic production of large quantities of RNA and its translation product(s).

The gene can be placed under the control of a promoter, ribosome binding site (for bacterial expression) and, optionally, an operator (collectively referred to herein as "control" elements), so that the DNA sequence encoding the desired E1 or E2 polypeptide is transcribed into RNA in the host cell transformed by a vector containing this expression construction. The coding sequence may or may not contain a signal peptide or leader sequence. With the present invention, both the naturally occurring signal peptides or heterologous sequences can be used. Leader sequences can be removed by the host in post-translational processing. *See, e.g.*, U.S. Patent Nos. 4,431,739; 4,425,437; 4,338,397. Such sequences include, but are not limited to, the tpa leader, as well as the honey bee mellitin signal sequence.

Other regulatory sequences may also be desirable which allow for regulation of expression of the protein sequences relative to the growth of the host cell. Such regulatory sequences are known to those of skill in the art, and examples include those which cause the expression of a gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Other types of regulatory elements may also be present in the vector, for example, enhancer sequences.

The control sequences and other regulatory sequences may be ligated to the coding sequence prior to insertion into a vector. Alternatively, the coding

sequence can be cloned directly into an expression vector which already contains the control sequences and an appropriate restriction site.

In some cases it may be necessary to modify the coding sequence so that it may be attached to the control sequences with the appropriate orientation; *i.e.*, to maintain the proper reading frame. It may also be desirable to produce mutants or analogs of the E1 or E2 protein. Mutants or analogs may be prepared by the deletion of a portion of the sequence encoding the protein, by insertion of a sequence, and/or by substitution of one or more nucleotides within the sequence. Techniques for modifying nucleotide sequences, such as site-directed mutagenesis, are well known to those skilled in the art. *See, e.g.*, Sambrook *et al., supra; DNA Cloning*, Vols. I and II, *supra; Nucleic Acid Hybridization, supra.*

The expression vector is then used to transform an appropriate host cell. A number of mammalian cell lines are known in the art and include immortalized cell lines available from the American Type Culture Collection (ATCC), such as, but not limited to, Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human hepatocellular carcinoma cells (e.g., Hep G2), as well as others. Similarly, bacterial hosts such as *E. coli, Bacillus subtilis,* and *Streptococcus spp.*, will find use with the present expression constructs. Yeast hosts useful in the present invention include *inter alia, Saccharomyces cerevisiae, Candida albicans, Candida maltosa, Hansenula polymorpha, Kluyveromyces fragilis, Kluyveromyces lactis, Pichia guillerimondii, Pichia pastoris, Schizosaccharomyces pombe* and *Yarrowia lipolytica*. Insect cells for use with baculovirus expression vectors include, *inter alia, Aedes aegypti,*

Autographa californica, *Bombyx mori*, *Drosophila melanogaster*, *Spodoptera frugiperda*, and *Trichoplusia ni*.

Depending on the expression system and host selected, the proteins of the present invention are produced by growing host cells transformed by an expression vector described above under conditions whereby the protein of interest is expressed. The selection of the appropriate growth conditions is within the skill of the art. The cells are then disrupted, using chemical, physical or mechanical means, which lyse the cells yet keep the HCV polypeptides substantially intact. Intracellular proteins can also be obtained by removing components from the cell wall or membrane, e.g., by the use of detergents or organic solvents, such that leakage of the E1 and/or E2 polypeptides occurs. Such methods are known to those of skill in the art and are described in, e.g., *Protein Purification Applications: A Practical Approach*, (E.L.V. Harris and S. Angal, Eds., 1990)

For example, methods of disrupting cells for use with the present invention include but are not limited to: sonication or ultrasonication; agitation; liquid or solid extrusion; heat treatment; freeze-thaw; desiccation; explosive decompression; osmotic shock; treatment with lytic enzymes including proteases such as trypsin, neuraminidase and lysozyme; alkali treatment; and the use of detergents and solvents such as bile salts, sodium dodecylsulphate, Triton, NP40 and CHAPS. The particular technique used to disrupt the cells is largely a matter of choice and will depend on the cell type in which the polypeptide is expressed, culture conditions and any pre-treatment used.

Following disruption of the cells, cellular debris is removed, generally by centrifugation, and the intracellularly produced E1 and/or E2 polypeptides are further purified, using standard purification techniques such as but not limited to, column chromatography, ion-exchange chromatography, size-exclusion chromatography, electrophoresis, HPLC, immunoadsorbent techniques, affinity chromatography, immunoprecipitation, and the like.

For example, one method for obtaining the intracellular HCV polypeptides of the present invention involves affinity purification, such as by immunoaffinity chromatography using anti-E1 and/or anti-E2 specific antibodies, or by lectin affinity chromatography. Particularly preferred lectin resins are those that recognize mannose moieties such as but not limited to resins derived from *Galanthus nivalis* agglutinin (GNA), *Lens culinaris* agglutinin (LCA or lentil lectin), *Pisum sativum* agglutinin (PSA or pea lectin), *Narcissus pseudonarcissus* agglutinin (NPA) and *Allium ursinum* agglutinin (AUA). The choice of a suitable affinity resin is within the skill in the art. After affinity purification, the E1 and E2 polypeptides can be further purified using conventional techniques well known in the art, such as by any of the techniques described above.

It may be desirable to produce E1/E2 complexes. Such complexes are readily produced by e.g., co-transfecting host cells with constructs encoding for the E1 and E2 truncated proteins. Co-transfection can be accomplished either in *trans* or *cis*, i.e., by using separate vectors or by using a single vector which bears both of the E1 and E2 genes. If done using a single vector, both genes can be driven by a single set of control elements or, alternatively, the genes can be present on the vector

in individual expression cassettes, driven by individual control elements. Following expression, the E1 and E2 proteins will spontaneously associate. Alternatively, the complexes can be formed by mixing the individual proteins together which have been produced separately, either in purified or semi-purified form, or even by mixing culture media in which host cells expressing the proteins, have been cultured. See, International Publication No. WO 96/04301, published February 15, 1996, for a description of such complexes.

The intracellularly produced E1 and E2 polypeptides of the present invention, complexes thereof, or the polynucleotides coding therefor, can be used for a number of diagnostic and therapeutic purposes. For example, the proteins and polynucleotides or antibodies generated against the same, can be used in a variety of assays, to determine the presence of reactive antibodies/and or E1 and E2 proteins in a biological sample to aid in the diagnosis of HCV disease.

The presence of antibodies reactive with the HCV polypeptides and, conversely, antigens reactive with antibodies generated thereto, can be detected using standard electrophoretic and immunodiagnostic techniques, including immunoassays such as competition, direct reaction, or sandwich type assays. Such assays include, but are not limited to, western blots; agglutination tests; enzyme-labeled and mediated immunoassays, such as ELISAs; biotin/avidin type assays; radioimmunoassays; immunoelectrophoresis; immunoprecipitation, etc. The reactions generally include revealing labels such as fluorescent, chemiluminescent, radioactive, or enzymatic labels or dye molecules, or other methods for detecting the

formation of a complex between the antigen and the antibody or antibodies reacted therewith.

Solid supports can be used in the assays such as nitrocellulose, in membrane or microtiter well form; polyvinylchloride, in sheets or microtiter wells; polystyrene latex, in beads or microtiter plates; polyvinylidine fluoride; diazotized paper; nylon membranes; activated beads, and the like.

Typically, the solid support is first reacted with the biological sample (or the E1 and/or E2 proteins), washed and then the antibodies, (or a sample suspected of containing antibodies), applied. After washing to remove any non-bound ligand, a secondary binder moiety is added under suitable binding conditions, such that the secondary binder is capable of associating selectively with the bound ligand. The presence of the secondary binder can then be detected using techniques well known in the art. Typically, the secondary binder will comprise an antibody directed against the antibody ligands. A number of anti-human immunoglobulin (Ig) molecules are known in the art (e.g., commercially available goat anti-human Ig or rabbit anti-human Ig). Ig molecules for use herein will preferably be of the IgG or IgA type, however, IgM may also be appropriate in some instances. The Ig molecules can be readily conjugated to a detectable enzyme label, such as horseradish peroxidase, glucose oxidase, Beta-galactosidase, alkaline phosphatase and urease, among others, using methods known to those of skill in the art. An appropriate enzyme substrate is then used to generate a detectable signal.

Alternatively, a "two antibody sandwich" assay can be used to detect the proteins of the present invention. In this technique, the solid support is reacted first with one or more of the

antibodies directed against E1 and/or E2, washed and then exposed to the test sample. Antibodies are again added and the reaction visualized using either a direct color reaction or using a labeled second antibody, such as an anti-immunoglobulin labeled with horseradish peroxidase, alkaline phosphatase or urease.

Assays can also be conducted in solution, such that the viral proteins and antibodies thereto form complexes under precipitating conditions. The precipitated complexes can then be separated from the test sample, for example, by centrifugation. The reaction mixture can be analyzed to determine the presence or absence of antibody-antigen complexes using any of a number of standard methods, such as those immunodiagnostic methods described above.

The E1 and/or E2 proteins, produced as described above, or antibodies to the proteins, can be provided in kits, with suitable instructions and other necessary reagents, in order to conduct immunoassays as described above. The kit can also contain, depending on the particular immunoassay used, suitable labels and other packaged reagents and materials (i.e. wash buffers and the like). Standard immunoassays, such as those described above, can be conducted using these kits.

The E1 and E2 polypeptides and polynucleotides encoding the polypeptides can also be used in vaccine compositions, individually or in combination, in e.g., prophylactic (i.e., to prevent infection) or therapeutic (to treat HCV following infection) vaccines. The vaccines can comprise mixtures of one or more of the E1 and E2 proteins (or nucleotide sequences encoding the proteins), such as E1 and E2 proteins derived from more than one viral isolate. The vaccine may also be administered in

conjunction with other antigens and immunoregulatory agents, for example, immunoglobulins, cytokines, lymphokines, and chemokines, including but not limited to IL-2, modified IL-2 (cys125→ser125), GM-CSF, IL-12, γ-interferon, IP-10, MIP1β and RANTES.

The vaccines will generally include one or more "pharmaceutically acceptable excipients or vehicles" such as water, saline, glycerol, ethanol, etc. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles.

A carrier is optionally present which is a molecule that does not itself induce the production of antibodies harmful to the individual receiving the composition. Suitable carriers are typically large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycollic acids, polymeric amino acids, amino acid copolymers, lipid aggregates (such as oil droplets or liposomes), and inactive virus particles. Such carriers are well known to those of ordinary skill in the art. Furthermore, the HCV polypeptide may be conjugated to a bacterial toxoid, such as toxoid from diphtheria, tetanus, cholera, etc.

Adjuvants may also be used to enhance the effectiveness of the vaccines. Such adjuvants include, but are not limited to: (1) aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, aluminum sulfate, etc.; (2) oil-in-water emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides (see below) or bacterial cell wall components), such as for example (a) MF59 (International Publication No. WO 90/14837), containing 5% Squalene, 0.5% Tween 80, and 0.5% Span 85 (optionally containing various amounts of

MTP-PE (see below), although not required) formulated into submicron particles using a microfluidizer such as Model 110Y microfluidizer (Microfluidics, Newton, MA), (b) SAF, containing 10% Squalane, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP (see below) either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and (c) Ribi™ adjuvant system (RAS), (Ribi Immunochem, Hamilton, MT) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (Detox™); (3) saponin adjuvants, such as Stimulon™ (Cambridge Bioscience, Worcester, MA) may be used or particle generated therefrom such as ISCOMs (immunostimulating complexes); (4) Complete Freunds Adjuvant (CFA) and Incomplete Freunds Adjuvant (IFA); (5) cytokines, such as interleukins (IL-1, IL-2, etc.), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), etc.; (6) detoxified mutants of a bacterial ADP-ribosylating toxin such as a cholera toxin (CT), a pertussis toxin (PT), or an *E. coli* heat-labile toxin (LT), particularly LT-K63 (where lysine is substituted for the wild-type amino acid at position 63) LT-R72 (where arginine is substituted for the wild-type amino acid at position 72), CT-S109 (where serine is substituted for the wild-type amino acid at position 109), and PT-K9/G129 (where lysine is substituted for the wild-type amino acid at position 9 and glycine substituted at position 129) (see, e.g., International Publication Nos. W093/13202 and W092/19265); and (7) other substances that act as immunostimulating agents to enhance the effectiveness of the composition.

Muramyl peptides include, but are not limited to, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acteyl-normuramyl-L-alanyl-D-isogluatme (nor-MDP), N-acetylmuramyl-L-alanyl-D-isogluatminyl-L-alanine-2-(1'-2'-dipalmitoyl-*sn*-glycero-3-huydroxyphosphoryloxy)-ethylamine (MTP-PE), etc.

Typically, the vaccine compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared. The preparation also may be emulsified or encapsulated in liposomes for enhanced adjuvant effect, as discussed above.

The vaccines will comprise a therapeutically effective amount of the E1 and/or E2 truncated proteins, or complexes of the proteins, or nucleotide sequences encoding the same, and any other of the above-mentioned components, as needed. By "therapeutically effective amount" is meant an amount of an E1 and/or E2 truncated protein which will induce a protective immunological response in the individual to which it is administered. Such a response will generally result in the development in the subject of a secretory, cellular and/or antibody-mediated immune response to the vaccine. Usually, such a response includes but is not limited to one or more of the following effects; the production of antibodies from any of the immunological classes, such as immunoglobulins A, D, E, G or M; the proliferation of B and T lymphocytes; the provision of activation, growth and differentiation signals to immunological cells; expansion of helper T cell, suppressor T cell, and/or cytotoxic T cell and/or $\gamma\delta$ T cell populations.

Preferably, the effective amount is sufficient to bring about treatment or prevention of disease symptoms. The exact amount necessary will

vary depending on the subject being treated; the age and general condition of the individual to be treated; the capacity of the individual's immune system to synthesize antibodies; the degree of protection desired; the severity of the condition being treated; the particular HCV polypeptide selected and its mode of administration, among other factors. An appropriate effective amount can be readily determined by one of skill in the art. A "therapeutically effective amount" will fall in a relatively broad range that can be determined through routine trials.

Once formulated, the vaccines are conventionally administered parenterally, e.g., by injection, either subcutaneously or intramuscularly. Additional formulations suitable for other modes of administration include oral and pulmonary formulations, suppositories, and transdermal applications. Dosage treatment may be a single dose schedule or a multiple dose schedule. The vaccine may be administered in conjunction with other immunoregulatory agents.

III. Experimental

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperatures, etc.), but some experimental error and deviation should, of course, be allowed for.

Materials and Methods

HCV Templates

A series of truncated E1 templates, shown in Figure 3, and E2 templates, shown in Figure 4, were generated using PCR. The appropriate 5' primer containing a methionine residue and an *NcoI* site was used along with 3' primers that had a termination codon following the designated envelope endpoint and finally, for E1, a *BamHI* site. Both oligos had non-specific sequences on the ends to facilitate more efficient digestions by *NcoI* and *BamHI* enzymes. Digested PCR fragments were ligated into *NcoI*/*BamHI*-digested pTM1 (Elroy-Stein and Moss, *Proc. Natl. Acad. Sci. USA* (1990) 87:6743-6747). The pTM1 vector contains the T7 promoter and the EMC leader proximal to the *NcoI* cloning site which corresponds to the first methionine residue encoded by the designated DNA. E2 templates were digested with *NcoI* and *AscI* and cloned into *NcoI*(partial)/*AscI*-pTM1-CE2 (Selby et al., *J. Gen. Virol.* (1993) 74:1103-1113) to generate the H clones where translations began at amino acid 1 and encode core, E1 and the designated E2 regions.

For the truncated E1 polypeptides, coding templates began with a methionine residue, followed by isoleucine and then amino acid 172. In particular, coding templates beginning with a methionine residue, followed by isoleucine and then amino acid 172 of the HCV polyprotein and continuing to amino acid 330, and clones of 10 amino acid increments through amino acid 380, were generated. Amino acids 173 through 191 correspond to the C-terminus of core which apparently serves a role as a signal sequence. Mature E1 is thought to begin at amino acid 192 of the polyprotein following signal sequence cleavage.

For the truncated E2 constructs, the methionine at position 364 was used as the N-terminus in the constructions. Amino acid 364 corresponds to the approximate start of the E2 signal peptide (Hijikata et al., *Proc. Natl. Acad. Sci. USA* (1991) 88:5547-5551; Ralston et al., *J. Virol.* (1993) 67:6753-6761). Mature E2 is thought to begin with amino acid 385. The staggered C-termini ranged from amino acid 661 through 1006. In particular, the clones terminated at amino acids 661, 699, 710, 715, 720, 725, 730, 760, 780, 807, 837, 906 and 1006.

In addition to those clones described above and shown in Figure 4, truncated E2 polypeptides, terminating at amino acids 500, 550, 590, 625, 655, and a construct terminating at amino acid 715 and including an additional deletion of the N-terminal hypervariable region, termed δ715, were also made as described above.

Intracellular Production of Truncated HCV E2

A truncated E2 molecule was expressed which had its C-terminus at amino acid 715_{lys} ("$E2_{715}$") as follows. A clone terminating at amino acid 715 was used to transfect a DHFR-deficient Chinese hamster ovary (CHO) cell line using techniques described in Spaete et al., *Virol.* (1992) 188:819-830. Following expression, CHO cells were lysed and the intracellularly produced $E2_{715}$ was purified by GNA agarose chromatography, followed by cation-exchange chromatography, as follows.

To lyse the cells, two volumes of lysis buffer (4% Triton X-100 in 0.1M Tris pH8, 1mM EDTA, 1 μg/ml pepstatin A and 1mM phenylmethyl-sulfonyl fluoride (PMSF)) were added to the CHO cells at 4°C. The mixture was homogenized in aliquots in a 40 ml Dounce homogenizer using tight-fit pestle B (20

strokes). The homogenate was spun at 12 K rpm for 20 min at 4°C. The supernatant was adjusted with water to 2% Triton/50mM Tris pH8 and homogenized again using pestle B (10 strokes). The homogenate was spun as above and the intracellularly produced $E2_{715}$ further purified from the supernatant as follows.

A GNA agarose column (4 ml bed volume, Vector Labs, Burlingame, CA) was pre-equilibrated with detergent buffer (2% Triton/50mM Tris pH8). The supernatant sample was applied to the column at 4°C and the column was washed with 5 bed-volumes of column buffer (0.1% Triton/20mM sodium phosphate pH6). The column was also washed with 5 bed-volumes of 1M NaCl. (Alternatively, the NaCl can be added to the detergent buffer to a concentration of 1M prior to loading the sample unto the column.) The E2 protein was eluted in 5 bed-volumes of methyl α-D-mannopyranoside (MMP) and 1M NaCl and 1.2 ml fractions collected.

Fractions containing the E2 protein were pooled and diluted with 2 volumes of S-Sepharose buffer (0.1% Triton/20mM sodium phosphate pH6). The pooled and diluted fractions were membrane-dialysed (Spectra/Por, molecular weight cut-off 1,000) against 4 L of S-Sepharse buffer overnight at 4°C with constant stirring. Following dialysis, the dialysate was applied to an S-Sepharose column (fast flow, Pharmacia, 4°C, 4 ml bed-volume, pre-equilibrated in S-Sepharose buffer). The column was washed in 5 bed-volumes of S-Sepharose buffer and 1 ml fractions eluted in 0.5M NaCl in S-Sepharose buffer. Fractions containing E2 were pooled and the column was washed with 1M NaCl in S-Sepharose buffer and re-equilibrate in S-Sepharose buffer.

E2 polypeptide δ715 (described above), as well as the E2 molecules terminating at amino acids

500, 550, 590, 625 and 655 were also produced as described above.

Production of Secreted, Truncated HCV E2

A truncated E2 molecule was made which had its C-terminus at amino acid 715_{lys} ("$E2_{715}$"). The truncated E2 molecule was expressed using a Chinese hamster ovary cell/dihydrofolate reductase (CHO/DHFR) expression system as described in Spaete et al., *Virol.* (1992) 188:819-830. Following expression, secreted $E2_{715}$ was purified for use in further experiments as described in International Publication No. WO 96/04301, published February 15, 1996.

E2 polypeptide δ715 (described above), as well as the E2 molecules terminating at amino acids 500, 550, 590, 625 and 655 were also produced as described above.

Intracellular Production of E1/E2 Complex

An E1/E2 complex, including complexed full-length E1 and E2, produced in HeLa cells, was isolated as follows. HeLa S3 cells were inoculated with purified high-titer vaccinia virus that expressed E1 and E2 at a multiplicity of infection of 5 pfu/cell, and the mixture was stirred at 37°C for 30 minutes. The infected cells were then transferred to a spinner flask containing 8 liters spinner medium and incubated for 3 days at 37°C. The cells were collected again by centrifugation and resuspended in hypotonic buffer (20mM HEPES, 10mM NaCl, 1mM NaCl, 1mM $MgCl_2$, 120 ml) on ice.

Following expression, HeLa cells were lysed with Triton X-100 and E1/E2 was isolated by GNA agarose chromatography followed by cation-exchange chromatography using the procedure used to purify the intracellularly expressed E2. The resulting material

was provided in buffer containing 0.05% Triton X-100. Reducing SDS-PAGE analysis showed it to have a relatively tight 55 kD band consistent with the presence of a large amount of mannose-type glycosylation. Purity was estimated at 33%. The complex was used as a control in the following experiments.

Example 1

Receptor Binding of Secreted and Intracellular E2

The GNA agarose-purified intracellular E2, and secreted E2, were used in binding assays as described in Rosa et al., *Proc. Natl. Acad. Sci. USA* (1996) 93:1759-1763 and International Publication No. WO 96/05513, published February 22, 1996. This assay assesses specific binding of the E2 proteins to human lymphoma T-cells (MOLT-4 cells) and the neutralization thereof, based on the cytofluorimetric assessment of sera that neutralize the binding of the antigens to the MOLT-4 cells.

As shown in Figure 5, the intracellular (internal) E2 bound to the cells approximately 30-fold more efficiently than the secreted counterpart.

Example 2

Immunoreactivity of Secreted and Intracellular E2

The immunoreactivity of the intracellularly produced truncated E2 protein was also compared to that of the secreted E2 protein using mouse monoclonal antibodies and human polyclonal sera.

A. Epitope Exposure Study of Intracellular and Secreted E2 Using Anti-E2 Monoclonal Antibodies.

Mouse monoclonal antibodies used in the assays were produced as follows. Mice were immunized with the immunogen specified in Table 1. Spleen cells

from immunized mice were obtained and fused with 2.5 x 10^7 NSO/2 mouse myeloma cells in 50% polyethylene glycol 4000 (Merck) using the procedure described by Kohler and Milstein, *Nature* (1975) 256:495-497. After the fusion, the cells were resuspended in HAT made with Dulbecco's medium, supplemented with nonessential amino acids, 10% fetal bovine serum (FBS) (Hyclone Laboratories, Salt Lake City, UT) 100 Ul/ml of penicillin, 100 μg/ml streptomycin, 2mM L-glutamine, 10^{-8}M hypoxanthine, 4 x 10^{-3}M thymidine (all reagents from Sigma). Aliquots of 100 μl were seeded on 96-well tissue culture plates (Nunc, DK 4000 Roskikle, Denmark). The plates were incubated at 37°C in moisturized atmosphere with 10% CO_2 in air. Ten days after fusion, supernatants from cultures exhibiting hybridoma growth were screened by hemagglutination for the production of anti-B antibodies. All positive cultures were expanded to 24-well plates and the cells were frozen under liquid nitrogen in a medium containing 90% (FCS and 10% dimethylsulfoxide (Merck). Hybridomas of interest were selected on the basis of their specificity, avidity, and intensity of the agglutination reaction and recloned twice by the limiting dilution technique.

To induce ascitic fluid, the recloned hybridomas were grown in the peritoneal cavity of BALB/C or F_2, (BALB/C X B10) mice, previously injected intraperitoneally with 0.5 ml of 2,6,10,14-tetramethylpenindecane (Pristane, from Sigma).

Isotypes of the antibodies were determined with a commercial kit, based on agglutination of sheep red blood cells coupled with rat monoclonal antibodies against mouse immunoglobulin isotype (Serotec, 22 Bankside, Oxford, England). The isotype, specificity and characterization of the monoclonal antibodies is shown in Table 1.

Table 1
HCV Monoclonal Antibodies List

Mab ID#	Isotype	Specificity	Characterization	Immunogen
5E5/H7	IgG1	anti-HCV e2	Conformational Ab	HeLa e1/e2 (aa 1-967)
2A3/B12	ND	anti-HCV e2	Conformational Ab	HeLa e1/e2 (aa 1-967)
5E9/D10	ND	anti-HCV e2	Conformational Ab	HeLa e1/e2 (aa 1-967)
3F5/H6	ND	anti-HCV e2	Conformational Ab	HeLa e1/e2 (aa 1-967)
3D5/C3	IgG1	anti-HCV e2	Anti-linear epitope Ab	HeLa e1/e2 (aa 1-967)
3E5-1	IgG1	anti-HCV e2	Anti-linear epitope Ab	Insect e2 (aa 404-661)
The conformational anti-e2 Mabs titers: 3E5/H7>3F5/H6>5E9/D10>B12				
472.2-5	ND	anti-HCV e2	Anti-hypervariable region	e2 HV peptide
6A1	IgG1	anti-HCV e2	Conformational Ab (blocks binding to MOLT4 receptor)	CHO e1/e2 (aa 1-967)
6A21	IgG1	anti-HCV e2	Conformational Ab (blocks binding to MOLT4 receptor)	

Epitope exposure of the secreted and intracellularly produced E2 proteins was determined using the monoclonal antibodies above as follows. Costar high- binding plates were loaded with 200 μl of purified E2 antigen (200 ng/well), as described in Table 2, which had been diluted in coating buffer. Plates were incubated overnight at room temperature and then washed 3 times with dH_2O. The plates were post coated with 300 μl of post coat solution and incubated for 1 hour at room temperature. Plates were aspirated and 300 μl of stability solution added to the plate. Plates were incubated for 1 hour at room temperature, aspirated and tapped several times. The plates were dried in a lyophilizer for at least 4 hours.

200 μl of prediluted (1:100) monoclonal antibodies, specified in Table 2, were added to the plates and the plates were incubated at 37°C for 1 hour. The plates were washed 5 times with wash buffer and 200 μl of 1:5 K conjugate goat anti-mouse IgG (H+L) Fab'2, added. Plates were incubated at 37°C for 1 hour and washed as above. The plates were developed using 200 μl of OPD Substrate. The results are shown in Table 2. As can be seen, the secreted E2 protein was only recognized by a monoclonal antibody against a linear epitope whereas the intracellularly (internal) produced E2 was recognized by monoclonal antibodies against both linear and conformational epitopes.

Table 2
Evaluation of Epitope Exposure by Murine Anti-E2 Monoclonal Antibodies

	Anti-E1 Mab NC 3D5/C3	Anti-E2 Mab Linear Epitope 3E5-1	Anti-E2 Mab Conformational Epitope 5E5/H7	Anti-E2 Mab Conformational Epitope 3F5/H6	Anti-E2 Mab Neutralizing Epitope 6A21
Internal CHOE2 (50%)	0.004	1.923	0.496	0.391	0.355
Secreted CHOE2 Dimer (80% Pure)	0.000	1.392	0.037	0.025	0.009
Secreted CHOE2 Monomer/Dimer (60% Pure)	0.007 0.001	1.833 1.605	0.161 0.122	0.115 0.067	0.140 0.051

NC = Negative control
Cut Off = 0.350 OD

B. Immunoreactivity Study of Secreted and Intracellular E2 Using HCV Serconversion.

In order to further determine the immunoreactivity of intracellularly produced truncated E2 versus the secreted E2, seroconversion panels were run using a commercial source of sera. 5 μl of each serum sample, diluted in 200 μl of sample diluent, were added to plates, prepared as described above, using the antigens identified in Table 3. The plates were incubated at 37°C for 1 hour and washed 5 times with wash buffer. 200 μl of conjugate goat anti-human IgG (H+L) Fab'2, diluted in conjugate diluent, was added and plates were incubated at 37°C for 1 hour. Plates were washed as above and developed using 200 μl of OPD Substrate.

Table 3
Evaluation of E2 ELISA Assay Sensitivity by Testing HCV Seroconversion Panel

	Internal CHOE2 50% Pure	Secreted CHOE2 Dimer (80% Pure)	Secreted CHOE2 Monomer/Dimer (60% Pure)
Sample	OD	OD	OD
PHV904-01	0.032	0.026	0.020
PHV904-02	0.040	0.041	0.028
PHV904-03	0.109	0.017	0.019
PHV904-04	0.505	0.048	0.029
PHV904-05	1.681	0.268	0.158
PHV904-06	1.095	0.152	0.082
PHV904-07	0.987	0.105	0.063

The intracellular E2 was significantly more sensitive than any of the secreted E2 proteins in detecting seroconversion. See, e.g., Table 3 which details the results from a typical assay.

The above results indicate that the intracellularly produced truncated E2 protein is more immunoreactive than the secreted counterpart. Thus, intracellular protein provides a better diagnostic reagent due to the enhanced immunoreactivity.

C. Immunoreactivity Study of Secreted and Intracellular E2 Using Immobilized HCV Antibodies.

In order to further determine the immunoreactivity of intracellularly produced truncated E2 versus the secreted E2, further studies were conducted using immobilized HCV antibodies. Antibodies used in these assays were monoclonal antibodies 6A21 and 3E5-1, both described in Table 1 above, as well as an IgG antibody preparation purified from serum of an HCV-infected patient, and specific for the hypervariable region at the N-terminus of E2.

In particular, antibody FF25931 was purified from patient serum through a Protein G affinity column and then conjugated to paramagnetic particles (Chiron Diagnostics, Walpole, MA) prior to use in the assays. Monoclonal antibodies 6A21 and 3E5-1 were also Protein G purified through a 5 ml gel (Pierce, Rockford, IL) and the antibodies covalently linked to magnetic latex particles (Bangs Laboratories, Fisher, IN) using 1-ethyl-3-C3-dimethylaminopropyl carbodimide hydrochloride (EDC) chemistry.

The detection reagent used in the assays was guinea pig polyclonal antisera raised against secreted $E2_{715}$, produced as described above. 1 ml of the polyclonal antisera was purified by passage through a 1 ml gel Protein A column (Pierce). Protein content

was determined by reading absorbance at 280 nm. The antibody was labeled with 2',6'-dimethyl-4'-(N-succinimidyloxycarboxnyl)phenyl-10-(3'-sulfopropyl)-acridinium-9-carboxylate (NHS-NSP-DMAE) (Chiron Diagnostics, Walpole, MA).

Both the solid-phase antibodies and the detection reagent were optimized and diluted in working buffer (50 mM Tris pH 8.0, 500 mM KCl, 1 mM EDTA, 1.75% BSA, 0.01% Tween-20).

Assays were conducted as follows. 100 µl sample were placed in a 75 x 12 mm polystyrene tube (Sarstedt, Newton, NC) and 100 µl of the solid-phase antibody added at 30 µg/assay. This was incubated at 37°C for 18 minutes. 100 µl of the detection reagent was added in an amount of $30x10^6$ relative light units (RLUs) per assay and the reaction allowed to proceed for 18 minutes at 37°C. The product was washed three times with phosphate bufferred saline, 0.1% Tween-20 and tubes were read using a Magic Lite Analyzer II (Chiron Diagnostics, Walpole, MA). Results are shown in Table 4.

The assay was repeated using the FF25931 antibody, immobilized on paramagnetic particles, as described above and detection was accomplished using monoclonal antibody 1G2/A7, specific for the hypervariable region at the N-terminus of E2, conjugated to NHS-NSP-DMAE. Results of this assay are shown in Table 5.

As can be seen in Table 4, monoclonal antibody 3E5-1 reacts with E2 truncations terminating at amino acid 550 and higher, both in supernatants (media) as well as the intracellular lysates. However, the 6A21 monoclonal antibody which blocks binding of E2 in the Molt-4 NOB assay, does not bind to E2 truncations terminating between 500 and 625. There is substantial immunoreactivity with molecules

terminating at amino acid 655 and higher. Thus, it appears that residues below 655 are required for the E2 to assume the right structure to bind to 6A21. E2 terminating at 661 also has high reactivity to 6A21, but E2 terminating at 715 has a substantially lower immunoreactivity to 6A21. This effect is particularly seen in supernatant.

As shown in Tables 4 and 5, the same effect is seen with antibody FF25931, an antibody specific to the hypervariable region at the N-terminus of E2. Truncations at 625, 655 and 661 were very active, but there was a drop with $E2_{715}$ both in the supernatant and in the media. The δ715 molecules were substantially inactive with all antibodies tested.

The data show that $E2_{655}$ and $E2_{661}$ are considerably immunoreactive.

Table 4			
Solid Phase Antibody:	3E5-1	6A21	FF25931
	supernatant	supernatant	supernatant
control	1047	1093	1771
500	1232	955	18033
550	1138476	1109	4682
590	1015661	955	4820
625	991775	1109	5282
655	775221	12043	99037
661	740509	10980	88920
715	764918	2803	29044
δ715	1032	1032	1602
	lysate	lysate	lysate
control	2449	1186	2726
500	1525	1078	5144
550	531177	1309	2618
590	480264	1324	2325
625	393624	1971	2110
655	389743	5421	58797
661	400492	6283	64141
715	492492	2356	22453
δ715	1571	1386	1771

Table 5	
Solid Phase Antibody:	FF25931
	supernatant
control	2079
500	53623
550	9948
590	9748
625	11689
655	261589
661	204959
	lysate
control	2911
500	8778
550	4389
590	3388
625	7069
655	212166
661	148749

Example 3

Immunization of Guinea Pigs with Secreted and Intracellular E2

The immunogenicity of secreted and intracellularly produced HCV $E2_{715}$ was determined in guinea pigs. Five groups of animals were immunized with the antigens described above, formulated with MF-75 and MTP-PE as an adjuvant. The internal HeLa E1/E2 was used as a control E2 preparation. Guinea pigs were immunized IM at 0,, 1 and 3 months with doses specified in Table 6, and sera from the guinea pigs was collected and pooled for further study.

Table 6. E2 Preps and doses used to immunize guinea pigs.

E2 Prep	Group #	Dose[a]
Internal CHO E2	5 6 7 8	80 μg 8.0 μg 0.8 μg 8.0, 2.0, 0.8 μg
Secreted CHO E2	9 10 11 12	80 μg 8.0 μg 0.8 μg 8.0, 2.0, 0.8 μg
HeLa Internal E1/E2	16	8.0, 2.0, 0.8 μg

[a]diluted in MF75-0 containing 50 μg MTP-PE for the first dose and 10 μg for subsequent doses on days 0, 30 and 90. Five animals were used per group.

As can be seen in Table 7, the internal CHO E2 preparation produced blocking antibodies that appeared to be as high or higher than those antibodies produced by HeLa E1/E2-immunized guinea pigs. The secreted CHO E2 preparations did not produce detectable blocking antibodies. These results suggest that intracellularly produced E2 is far superior to the secreted extracellular form in inducing neutralizing antibodies.

Table 7. Blocking antibodies to the putative receptor in immunized guinea pigs.

E2 Prep	Group #	Dose	NOB (E2-1a)	NOB (E2-1b)
Internal CHO E2	5	80 μg	700	80
	6	8.0 μg	1000	ND
	7	0.8 μg	1500	ND
	8	8, 2, 0.8 μg	1000	100
Secreted CHO E2	9	80 μg	0	ND
	10	8.0 μg	0	ND
	11	0.8 μg	0	ND
	12	8, 2, 0.8 μg	0	ND
HeLa E1/E2	16	8, 2, 0.8 μg	600	400

Also as shown in Table 7, the internal E2 antigen induced NOB titers to the HCV1a antigen in contrast to the lack of neutralizing antibodies induced by the secreted E2 antigen. Furthermore, guinea pigs immunized with the internal E2 antigen also developed antibodies that could cross-neutralize HCV1b E2 binding to the T-cell line used.

Thus, methods for obtaining intracellularly expressed E1 and E2 polypeptides are disclosed, as are methods of using the same. Although preferred embodiments of the subject invention have been described in some detail, it is understood that obvious variations can be made without departing from the spirit and the scope of the invention as defined by the appended claims.

We Claim:

1. A method for isolating a hepatitis C virus (HCV) E1 polypeptide that lacks a portion of its C-terminus beginning at about amino acid 370 but not extending beyond about amino acid 300, numbered with reference to the HCV1 E1 amino acid sequence, said method comprising:

(a) providing a population of host cells transformed with a polynucleotide comprising a coding sequence for said HCV E1 polypeptide, wherein said coding sequence is operably linked to control elements such that said coding sequence can be transcribed and translated in the host cell;

(b) culturing said population of cells under conditions whereby said HCV E1 polypeptide is expressed intracellularly;

(c) disrupting said host cells; and

(d) isolating said HCV E1 polypeptide from said disrupted cells.

2. The method of claim 1, wherein said HCV E1 polypeptide is truncated after about amino acid 360, numbered with reference to the HCV1 E1 amino acid sequence.

3. The method of claims 1 or 2, wherein said isolating is done using affinity chromatography.

4. The method of claim 3, wherein said affinity chromatography is GNA agarose chromatography.

5. A method for isolating a hepatitis C virus (HCV) E2 polypeptide that lacks a portion of its C-terminus beginning at about amino acid 730 but not extending beyond about amino acid 500, numbered with

reference to the HCV1 E2 amino acid sequence, said method comprising:

(a) providing a population of host cells transformed with a polynucleotide comprising a coding sequence for said HCV E2 polypeptide, wherein said coding sequence is operably linked to control elements such that said coding sequence can be transcribed and translated in the host cell;

(b) culturing said population of cells under conditions whereby said HCV E2 polypeptide is expressed intracellularly; (c) disrupting said host cells; and

(d) isolating said HCV E2 polypeptide from said disrupted cells.

6. The method of claim 5, wherein said HCV E2 polypeptide lacks at least a portion of its C-terminus beginning at about amino acid 725 but not extending beyond about amino acid 625, numbered with reference to the HCV1 E2 amino acid sequence.

7. The method of claim 6, wherein said HCV E2 polypeptide is truncated after about amino acid 715, numbered with reference to the HCV1 E2 amino acid sequence.

8. The method of claim 6, wherein said HCV E2 polypeptide is truncated after about amino acid 661, numbered with reference to the HCV1 E2 amino acid sequence.

9. The method of claim 6, wherein said HCV E2 polypeptide is truncated after about amino acid 655, numbered with reference to the HCV1 E2 amino acid sequence.

10. The method of any of claims 5-9, wherein said isolating is done using affinity chromatography.

11. The method of claim 10, wherein said affinity chromatography is GNA agarose chromatography.

12. An HCV E1 polypeptide produced by the method of any of claims 1-4.

13. An HCV E2 polypeptide produced by the method of any of claims 5-11.

14. A composition comprising a pharmaceutically acceptable excipient and an HCV E1 polypeptide according to claim 12.

15. A composition comprising a pharmaceutically acceptable excipient and an HCV E2 polypeptide according to claim 13.

16. A method of preparing a composition comprising combining an HCV E1 polypeptide according to claim 12 with a pharmaceutically acceptable excipient.

17. A method of preparing a composition comprising combining an HCV E2 polypeptide according to claim 13 with a pharmaceutically acceptable excipient.

18. Use of an HCV E1 polypeptide according to claim 12 for the manufacture of a medicament useful for detecting the presence or absence of HCV infection in a subject suspected of having an HCV infection.

19. Use of an HCV E2 polypeptide according to claim 13 for the manufacture of a medicament useful for detecting the presence or absence of HCV infection in a subject suspected of having an HCV infection.

20. An immunodiagnostic test kit for detecting HCV infection, said test kit comprising an HCV E1 polypeptide according to claim 12 and instructions for conducting the immunodiagnostic test.

21. An immunodiagnostic test kit for detecting HCV infection, said test kit comprising an HCV E2 polypeptide according to claim 13 and instructions for conducting the immunodiagnostic test.

```
170 METILECYSSERPHESERILEPLIELEULEUALALEULEUSERCYSLEUTHRVALPROALA
    ATGATTTGCTCTTTCTCTATCTTCCTTCTGGCCCTGCTCTCTTGCTTGACTGTGCCCGCT
    TACTAAACGAGAAAGAGATAGAAGGAAGACCGGGACGAGAGAACGAACTGACACGGGCGA

          Mature E1
190 SERALATYRGLNVALARGASNSERTHRGLYLEUTYRHISVALTHRASNASPCYSPROASN
    TCGGCCTACCAAGTGCGCAACTCCACGGGGCTCTACCACGTCACCAATGATTGCCCTAAC
    AGCCGGATGGTTCACGCGTTGAGGTGCCCCGAGATGGTGCAGTGGTTACTAACGGGATTG

210 SERSERILEVALTYRGLUALAALAASPALAILELEUHISTHRPROGLYCYSVALPROCYS
    TCGAGTATTGTGTACGAGGCGGCCGATGCCATCCTGCACACTCCGGGGTGCGTCCCTTGC
    AGCTCATAACACATGCTCCGCCGGCTACGGTAGGACGTGTGAGGCCCCACGCAGGGAACG

230 VALARGGLUGLYASNALASERARGCYSTRPVALALAMETTHRPROTHRVALALATHRARG
    GTTCGTGAGGGCAACGCCTCGAGGTGTTGGGTGGCGATGACCCCTACGGTGGCCACCAGG
    CAAGCACTCCCGTTGCGGAGCTCCACAACCCACCGCTACTGGGGATGCCACCGGTGGTCC

250 ASPGLYLYSLEUPROALATHRGLNLEUARGARGHISILEASPLEULEUVALGLYSERALA
    GATGGCAAACTCCCCGCGACGCAGCTTCGACGTCACATCGATCTGCTTGTCGGGAGCGCC
    CTACCGTTTGAGGGGCGCTGCGTCGAAGCTGCAGTGTAGCTAGACGAACAGCCCTCGCGG

270 THRLEUCYSSERALALEUTYRVALGLYASPLEUCYSGLYSERVALPHELEUVALGLYGLN
    ACCCTCTGTTCGGCCCTCTACGTGGGGGACCTCTGCGGGTCTGTCTTTCTTGTCGGCCAA
    TGGGAGACAAGCCGGGAGATGCACCCCCTGGAGACGCCCAGACAGAAAGAACAGCCGGTT

290 LEUPHETHRPHESERPROARGARGHISTRPTHRTHRGLNGLYCYSASNCYSSERILETYR
    CTGTTTACCTTCTCTCCCAGGCGCCACTGGACGACGCAAGGTTGCAATTGCTCTATCTAT
    GACAAATGGAAGAGAGGGTCCGCGGTGACCTGCTGCGTTCCAACGTTAACGAGATAGATA

310 PROGLYHISILETHRGLYHISARGMETALATRPASPMETMETMETMETASNTRPSERPROTHR
    CCCGGCCATATAACGGGTCACCGCATGGCATGGGATATGATGATGAACTGGTCCCCTACG
    GGGCCGGTATATTGCCCAGTGGCGTACCGTACCCTATACTACTACTTGACCAGGGGATGC

330 THRALALEUVALMETALAGLNLEULEUARGILEPROGINALAILELEUASPMETILEALA
    ACGGCGTTGGTAATGGCTCAGCTGCTCCGGATCCCACAAGCCATCTTGGACATGATCGCT
    TGCCGCAACCATTACCGAGTCGACGAGGCCTAGGGTGTTCGGTAGAACCTGTACTAGCGA

                                        C-terminal Anchor
350 GLYALAHISTRPGLYVALLEUALAGLYILEALATYRPHESERMETVALGLYASNTRPALA
    GGTGCTCACTGGGGAGTCCTGGCGGGCATAGCGTATTTCTCCATGGTGGGGAACTGGGCG
    CCACGAGTGACCCCTCAGGACCGCCCGTATCGCATAAAGAGGTACCACCCCTTGACCCGC

370 LYSVALLEUVALVALLEULEULEUPHEALAGLYOP
    AAGGTCCTGGTAGTGCTGCTGCTATTTGCCGGCTGA
    TTCCAGGACCATCACGACGACGATAAACGGCCGACT
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FIG. 1

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364  METVALGLYASNTRPALALYSVALLEUVALVALLEULEULEUPHEALAGLYVALASPALA
     ATGGTGGGGAACTGGGCGAAGGTCCTGGTAGTGCTGCTGCTATTTGCCGGCGTCGACGCG
     TACCACCCCTTGACCCGCTTCCAGGACCATCACGACGACGATAAACGGCCGCAGCTGCGC

        MATURE E2
384  GLUTHRHISVALTHRGLYGLYSERALAGLYHISTHRVALSERGLYPHEVALSERLEULEU
     GAAACCCACGTCACCGGGGGAAGTGCCGGCCACACTGTGTCTGGATTTGTTAGCCTCCTC
     CTTTGGGTGCAGTGGCCCCCTTCACGGCCGGTGTGACACAGACCTAAACAATCGGAGGAG

404  ALAPROGLYALALYSGINASNVALGINLEULLEASNTHRASNGLYSERTRPHISLEUASN
     GCACCAGGCGCCAAGCAGAACGTCCAGCTGATCAACACCAACGGCAGTTGGCACCTCAAT
     CGTGGTCCGCGGTTCGTCTTGCAGGTCGACTAGTTGTGGTTGCCGTCAACCGTGGAGTTA

424  SERTHRALALEUASNCYSASNASPSERLEUASNTHRGLYTRPLEUALAGLYLEUPHETYR
     AGCACGGCCCTGAACTGCAATGATAGCCTCAACACCGGCTGGTTGGCAGGGCTTTTCTAT
     TCGTGCCGGGACTTGACGTTACTATCGGAGTTGTGGCCGACCAACCGTCCCGAAAAGATA

444  HISHISLYSPHEASNSERSERGLYCYSPROGLUARGLEUALASERCYSARGPROLEUTHR
     CACCACAAGTTCAACTCTTCAGGCTGTCCTGAGAGGCTAGCCAGCTGCCGACCCCTTACC
     GTGGTGTTCAAGTTGAGAAGTCCGACAGGACTCTCCGATCGGTCGACGGCTGGGGAATGG

464  ASPPHEASPGLNGLYTRPGLYPROILESERTYRALAASNGLYSERGLYPROASPGLNARG
     GATTTTGACCAGGGCTGGGGCCCTATCAGTTATGCCAACGGAAGCGGCCCCGACCAGCGC
     CTAAAACTGGTCCCGACCCCGGGATAGTCAATACGGTTGCCTTCGCCGGGGCTGGTCGCG

484  PROTYRCYSTRPHISTYRPROPROLYSPROCYSGLYILEVALPROALALYSSERVALCYS
     CCCTACTGCTGGCACTACCCCCCAAAACCTTGCGGTATTGTGCCCGCGAAGAGTGTGTGT
     GGGATGACGACCGTGATGGGGGGTTTTGGAACGCCATAACACGGGCGCTTCTCACACACA
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FIG. 2A

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504  GLYPROVALTYRCYSPHETHRPROSERPROVALVALVALGLYTHRTHRASPARGSERGLY
     GGTCCGGTATATTGCTTCACTCCCAGCCCCGTGGTGGTGGGAACGACCGACAGGTCGGGC
     CCAGGCCATATAACGAAGTGAGGGTCGGGGCACCACCACCCTTGCTGGCTGTCCAGCCCG

524  ALAPROTHRTYRSERTRPGLYGLUASNASPTHRASPVALPHEVALLEDASNASNTHRARG
     GCGCCCACCTACAGCTGGGGTGAAAATGATACGGACGTCTTCGTCCTTAACAATACCAGG
     CGCGGGTGGATGTCGACCCCACTTTTACTATGCCTGCAGAAGCAGGAATTGTTATGGTCC

544  PROPROLEUGLYASNTRPPHEGLYCYSTHRTRPMETASNSERTHRGLYPHETHRLYSVAL
     CCACCGCTGGGCAATTGGTTCGGTTGTACCTGGATGAACTCAACTGGATTCACCAAAGTG
     GGTGGCGACCCGTTAACCAAGCCAACATGGACCTACTTGAGTTGACCTAAGTGGTTTCAC

564  CYSGLYALAPROPROCYSVALILEGLYGLYALAGLYASNASNTHRLEUHISCYSPROTHR
     TGCGGAGCGCCTCCTTGTGTCATCGGAGGGGCGGGCAACAACACCCTGCACTGCCCCACT
     ACGCCTCGCGGAGGAACACAGTAGCCTCCCCGCCCGTTGTTGTGGGACGTGACGGGGTGA

584  ASPCYSPHEARGLYSHISPROASPALATHRTYRSERARGCYSGLYSERGLYPROTRPILE
     GATTGCTTCCGCAAGCATCCGGACGCCACATACTCTCGGTGCGGCTCCGGTCCCTGGATC
     CTAACGAAGGCGTTCGTAGGCCTGCGGTGTATGAGAGCCACGCCGAGGCCAGGGACCTAG

604  THRPROARGCYSLEUVALASPTYRPROTYRARGLEUTRPHISTYRPROCYSTHRILEASN
     ACACCCAGGTGCCTGGTCGACTACCCGTATAGGCTTTGGCATTATCCTTGTACCATCAAC
     TGTGGGTCCACGGACCAGCTGATGGGCATATCCGAAACCGTAATAGGAACATGGTAGTTG

624  TYRTHRILEPHELYSILEARGMETTYRVALGLYGLYVALGLUHISARGLEUGLUALAALA
     TACACCATATTTAAAATCAGGATGTACGTGGGAGGGGTCGAACACAGGCTGGAAGCTGCC
     ATGTGGTATAAAITTTAGTCCTACATGCACCCTCCCCAGCTTGTGTCCGACCTTCGACGG
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FIG. 2B

644 CYSASNTRPTHRARGGLYGLUARGCYSASPLEUGLUASPARGASPARGSERGLULEUSER
TGCAACTGGACGCGGGGCGAACGTTGCGATCTGGAAGATAGGGACAGGTCCGAGCTCAGC
ACGTTGACCTGCGCCCCGCTTGCAACGCTAGACCTTCTATCCCTGTCCAGGCTCGAGTCG

664 PROLEULEULEUTHRTHRTHRGLNTRPGLNVALLEUPROCYSSERPHETHRTHRLEUPRO
CCGTTACTGCTGACCACTACACAGTGGCAGGTCCTCCCGTGTTCCTTCACAACCCTGCCA
GGCAATGACGACTGGTGATGTGTCACCGTCCAGGAGGGCACAAGGAAGTGTTGGCACGGT

684 ALALEUSERTHRGLYLEUILEHISLEUHISGLNASNILEVALASPVALGLNTYRLEUTYR
GCCTTGTCCACCGGCCTCATCCACCTCCACCAGAACATTGTGGACGTGCAGTACTTGTAC
CGGAACAGGTGGCCGGAGTAGGTGGAGGTGGTCTTGTAACACCTGCACGTCATGAACATG

704 GLYVALGLYSERSERILEALASERTRPALAILELYSTRPGLUTYRVALVAILEULEUPHE
GGGGTGGGGTCAAGCATCGCGTCCTGGGCCATTAAGTGGGAGTACGTCGTCCTCCTGTTC
CCCCACCCCAGTTCGTAGCGCAGGACCCGGTAATTCACCCTCATGCAGCAGGAGGACAAG

C-terminal Anchor

724 LEULEULEUALAASPALAARGVALCYSSERCYSLEUTRPMETMETLEULEUILESERGLN
CTTCTGCTTGCAGACGCGCGCGTCTGCTCCTGCTTGTGGATGATGCTACTCATATCCCAA
GAAGACGAACGTCTGCGCGCGCAGACGAGGACGAACACCTACTACGATGAGTATAGGGTT

P7

744 ALAGLUALAALALEUGLUASNLEUVALILELEUASNALAALASERLEUALAGLYTHRHIS
GCGGAAGCGGCTTTGGAGAACCTCGTAATACTTAATGCAGCATCCCTGGCCGGGACGCAC
CGCCTTCGCCGAAACCTCTTGGAGCATTATGAATTACGTCGTAGGGACCGGCCCTGCGTG

764 GLYLEUVALSERPHELEUVALPHEPHECYSPHEALATRPTYRLEULYSGLYLYSTRPVAL
GGTCTTGTATCCTTCCTCGTGTTCTTCTGCTTTGCATGGTATCTGAAGGGTAAGTGGGTG
CCAGAACATAGGAAGGAGCACAAGAAGACGAAACGTACCATAGACTTCCCATTCACCCAC

FIG. 2C

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784  PROGLYALAVALTYRTHRPHETYRGLYMETTRPPROLEULEULEULEULEUALALEU
     CCCGGAGCGGTCTACACCTTCTACGGGATGTGGCCTCTCCTCCTGCTCCTGTTGGCGTTG
     GGGCCTCGCCAGATGTGGAAGATGCCCTACACCGGAGAGGAGGACGAGGACAACCGCAAC

                          NS2
804  PROGLNARGALATYRALALEUASPTHRGLUVALALAALASERCYSGLYGLYVALVALLEU
     CCCCAGCGGGCGTACGCGCTGGACACGGAGGTGGCCGCGTCGTGTGGCGGTGTTGTTCTC
     GGGGTCGCCCGCATGCGCGACCTGTGCCTCCACCGGCGCAGCACACCGCCACAACAAGAG

824  VALGLYLEUMETALALEUTHRLEUSERPROTYRTYRLYSARGTYRILESERTRPCYSLEU
     GTCGGGTTGATGGCGCTAACTCTGTCACCATATTACAAGCGCTATATCAGCTGGTGCTTG
     CAGCCCAACTACCGCGATTGAGACAGTGGTATAATGTTCGCGATATAGTCGACCACGAAC

844  TRPTRPLEUGLNTYRPHELEUTHRARGVALGLUALAGLNLEUHISVALTRPILEPROPRO
     TGGTGGCTTCAGTATTTTCTGACCAGAGTGGAAGCGCAACTGCACGTGTGGATTCCCCCC
     ACCACCGAAGTCATAAAAGACTGGTCTCACCTTC-CGTTGACGTGCACACCTAAGGGGGG

864  LEUASNVALARGGLYGLYARGASPALAVALILELEULEUMETCYSALAVALHISPROTHR
     CTCAACGTCCGAGGGGGGCGCGACGCCGTCATCTTACTCATGTGTGCTGTACACCCGACT
     GAGTTGCAGGCTCCCCCCGCGCTGCGGCAGTAGAATGAGTACACACGACATGTGGGCTGA

884  LEUVALPHEASPILETHRLYSLEULEULEUALAVALPHEGLYPROLEUTRPILELEUGLN
     CTGGTATTTGACATCACCAAATTGCTGCTGGCCGTCTTCGGACCCCTTTGGATTCTTCAA
     GACCATAAACTGTAGTGGTTTAACGACGACCGGCAGAAGCCTGGGGAAACCTAAGAAGTT

904  ALASERLEULEULYSVALPROTYRPHEVALARGVALGLNGLYLEULEUARGPHECYSALA
     CCCAGTTTGCTTAAAGTACCCTACTTTGTGCGCGTCCAAGGCCTTCTCCGGTTCTGCGCG
     CGGTCAAACGAATTTCATGGGATGAAACACGCGCAGGTTCCGGAAGAGGCCAAGACGCGC
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FIG. 2D

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924  LEUALAARGLYSMETILEGLYGLYHISTYRVALGLNMETVALILEILELYSLEUGLYALA
     TTAGCGCGGAAGATGATCGGAGGCCATTACGTGCAAATGGTCATCATTAAGTTAGGGGCG
     AATCGCGCCTTCTACTAGCCTCCGGTAATGCACGTTTACCAGTAGTAATTCAATCCCCGC

944  LEUTHRGLYTHRTYRVALTYRASNHISLEUTHRPROLEUARGASPTRPALAHISASNGLY
     CTTACTGGCACCTATGTTTATAACCATCTCACTCCTCTTCGGGACTGGGCGCACAACGGC
     GAATGACCGTGGATACAAATATTGGTAGAGTGAGGAGAAGCCCTGACCCGCGTGTTGCCG

964  LEUARGASPLEUALAVALALAVALGLUPROVALVALPHESERGLNMETGLUTHRLYSLEU
     TTGCGAGATCTGGCCGTGGCTGTAGAGCCAGTCGTCTTCTCCCAAATGGAGACCAAGCTC
     AACGCTCTAGACCGGCACCGACATCTCGGTCAGCAGAAGAGGGTTTACCTCTGGTTCGAG

984  ILETHRTRPGLYALAASPTHRALAALACYSGLYASPILEILEASNGLYLEUPROVALSER
     ATCACGTGGGGGGCAGATACCGCCGCGTGCGGTGACATCATCAACGGCTTGCCTGTTTCC
     TAGTGCACCCCCCGTCTATGGCGGCGCACGCCACTGTAGTAGTTGCCGAACGGACAAAGG

1004 ALAARGARGGLYARGGLUILELEULEUGLYPROALAASPGLYMETVALSERLYSGLYTRP
     GCCCGCAGGGGCCGGGAGATACTGCTCGGGCCAGCCGATGGAATGGTCTCCAAGGGTTGG
     CGGGCGTCCCCGGCCCTCTATGACGAGCCCGGTCGGCTACCTTACCAGAGGTTCCCAACC

1024 ARGLEULEU
     AGGTTGCTG
     TCCAACGAC
```

FIG. 2E

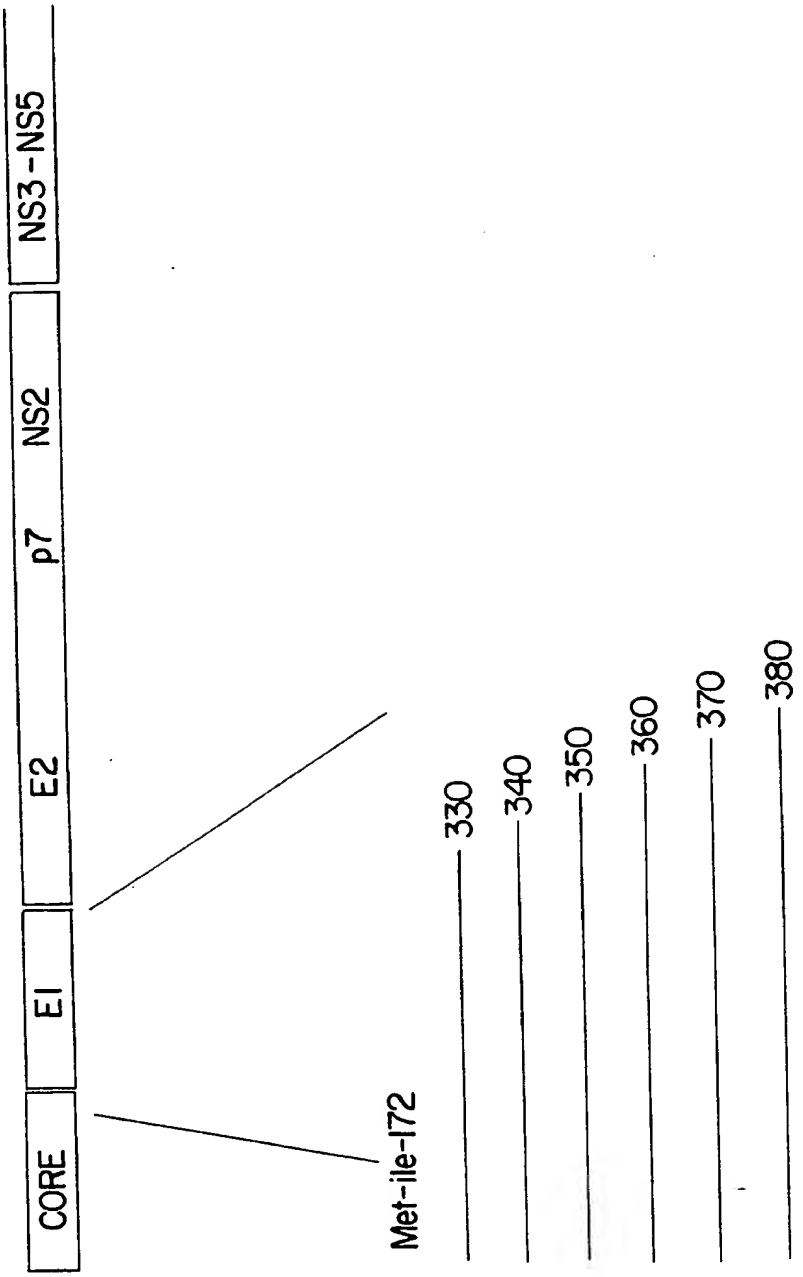

FIG. 3

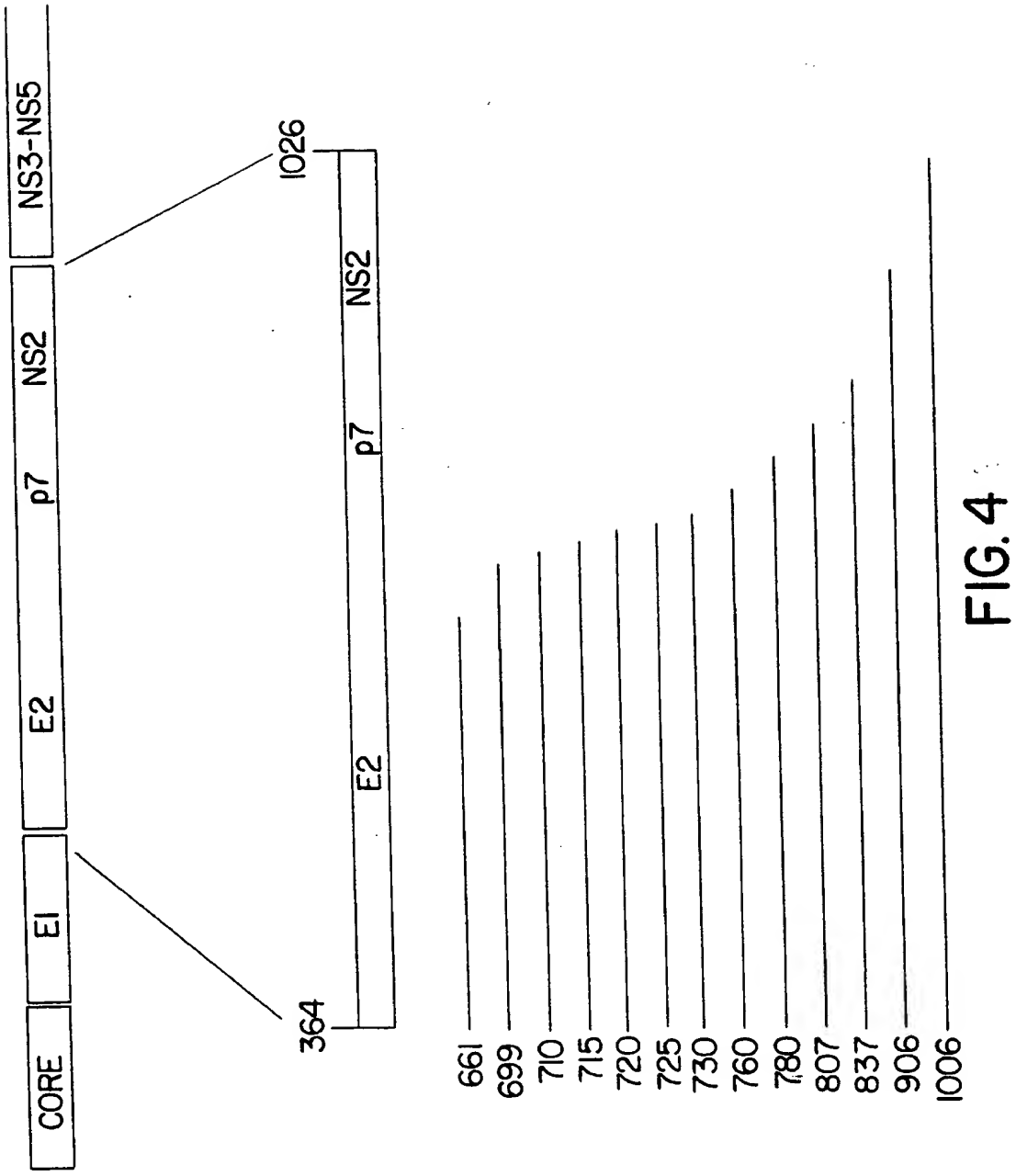
CORE
E1
E2
p7
NS2
NS3-NS5
364
1026
E2
p7
NS2
661
699
710
715
720
725
730
760
780
807
837
906
1006

FIG. 4

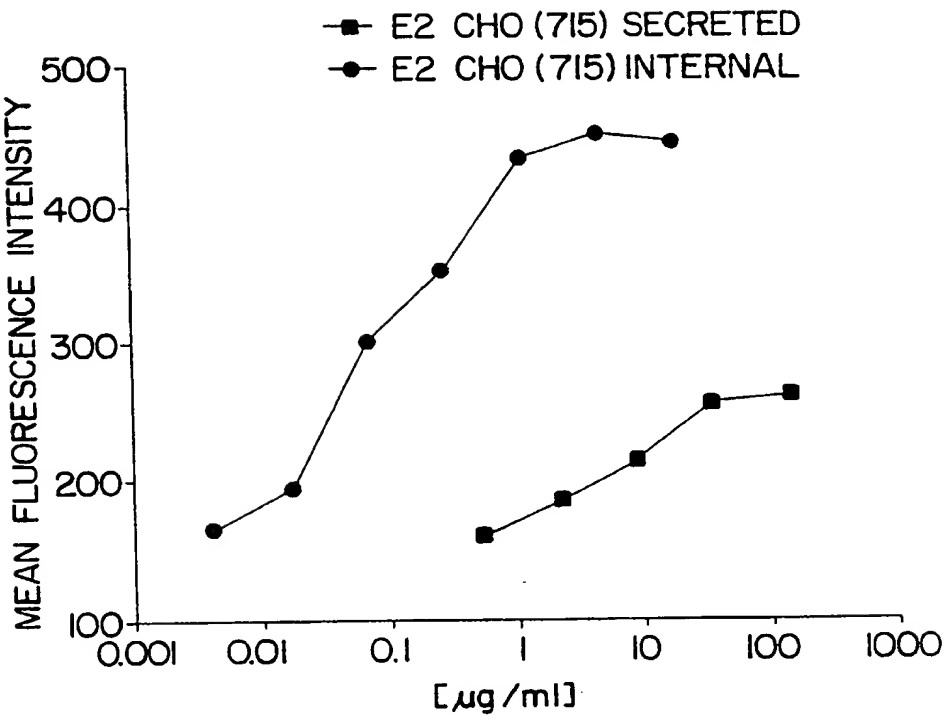
E2 CHO (715) SECRETED
E2 CHO (715) INTERNAL
MEAN FLUORESCENCE INTENSITY
500
400
300
200
100
0.001
0.01
0.1
1
10
100
1000
[µg/ml]

FIG. 5